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Techniques for Assessment of Teratologic Effects: Embryo Culture

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A simple method is described for growing rat embryos *in vitro* for 48 hr from head-fold to early limb-bud stages at rates of development and protein synthesis indistinguishable from those *in vivo*. Culture of the embryos can be continued for longer periods but at a reduced growth rate. Preheating the culture serum to 56°C for 30 min improves embryonic development, but raising the culture temperature 2-3°C or exposing the presomite embryos to 20% O_2 (160 mm Hg) causes malformations, particularly of the brain and spinal cord. The value of such culture methods for teratology is briefly discussed.

To be of value for assessing teratological effects, embryo culture methods must be capable of supporting normal development of post-implantation embryos during the period of organogenesis, and be sufficiently simple and cheap for routine and fairly large-scale testing. So far, these criteria have been met most successfully with the embryos of rodents, particularly rats and mice. Figure 1 shows the periods of post-implantation rat development that can now be maintained in culture. The younger embryos at primitive-streak stage (8-9 days gestation) can be grown almost to the 40-somite stage, equivalent to almost 4 days growth *in vivo*. The period is progressively shortened with increasing age at explantation, and embryos explanted at 13-14 days gestation show further development equivalent only to about 1 day *in vivo*.

Differentiation and organogenesis of the embryos *in vitro* usually resembles very closely that *in vivo* at all times up to about day 15. But growth, as measured by total protein synthesis, has often been found to be retarded (*1-4*). In the older embryos this probably results from a failure of the allantoic placenta to develop in culture, and until methods are devised for supporting placental growth, it seems unlikely that normal rates of embryo/fetal growth will be maintained in culture much beyond the time (about 11 days of gestation) at which the allantoic circulation becomes functional. However, the younger embryos are not subject to this limitation, and some recent work that I have been doing in collaboration with

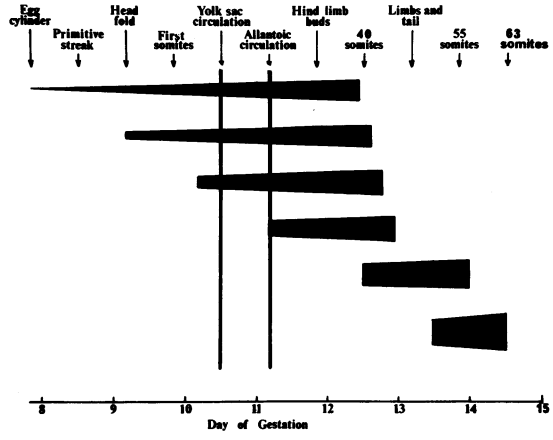

FIGURE 1. Periods of development (represented by black areas) of rat embryos in culture. The vertical lines indicate the times at which the yolk sac and allantoic blood circulations are established.

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my colleagues Pat Coppola and David Cockroft in Cambridge has concentrated on improving the culture methods for these stages with the aim of attaining growth rates similar to those *in vivo* (*5,6*).

Comparison of Growth *in Vitro* and *in Vivo*

Embryos at head-fold stage were obtained from rats of CFHB strain during the morning of day 10 of gestation (9½ days *post coitum*). To assess how closely growth *in vitro* resembled that *in vivo*, comparisons were made between embryos from the two uterine horns of each rat (Fig. 2). The rat was anaesthetized with ether, and one horn of the uterus drawn out through a small incision in the abdominal wall. This horn and its blood vessels were then ligated and cut free and the embryos explanted for culturing. Care was taken at all stages of the operation to avoid disturbing the uterine horn and blood vessels of the opposite side, and later comparison with unoperated animals confirmed that the operation had no effect on the embryos left to develop *in vivo*.

The explanted embryos were cultured with the visceral yolk-sac and ectoplacental cone intact but with the Reichert membrane torn open (Fig. 3). At this stage the neural folds were just beginning to appear but no other organs had yet formed. The embryos were incubated at 38°C in small cylindrical stoppered bottles (Fig. 4) rotated continuously at 40-50 rpm for periods of 32 or 48 hr. The bottles were of 30 ml capacity, containing four embryos and 4 ml of culture serum; in some experiments the embryos were transferred after 24 hr of incubation to bottles of 60 ml capacity containing 8 ml of serum. The culture serum was obtained from CFHB rats, without regard to the sex or age of the animals, and pooled before use. The serum was always prepared from blood certrifuged immediately after extraction from the rat before clotting had occurred (*7,8*) and was preheated (56°C for 30 min) before being added to the culture bottles. The gas phase was 5% O_2-5% CO_2-90% N_2 for the first 22-24 hr, followed by 20% O_2-5% CO_2-75% N_2. Some of the cultures received a further gassing at 32 hr with 20% O_2-5% CO_2-75% N_2 or 40% O_2-5% CO_2-55% N_2.

After 32 hr, all the embryos in culture had a good yolk-sac blood circulation. Those embryos with 17 or more somites, and an occasional embryo with 16 somites, also showed a rudimentary blood circulation in the allantois. No significant

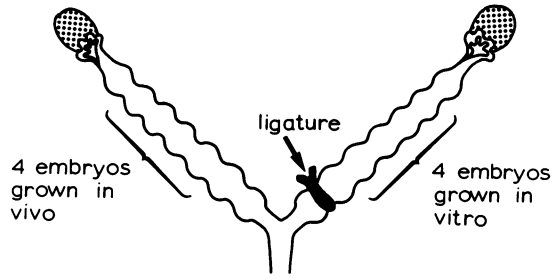

FIGURE 2. Selection of embryos from the two uterine horns for comparison of growth *in vivo* and *in vitro*.

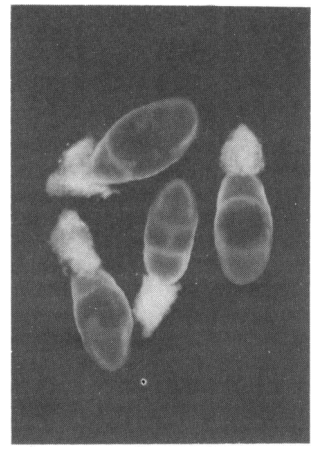

FIGURE 3. Head-fold stage rat embryos as explanted at 9½ days of gestation. ×10.

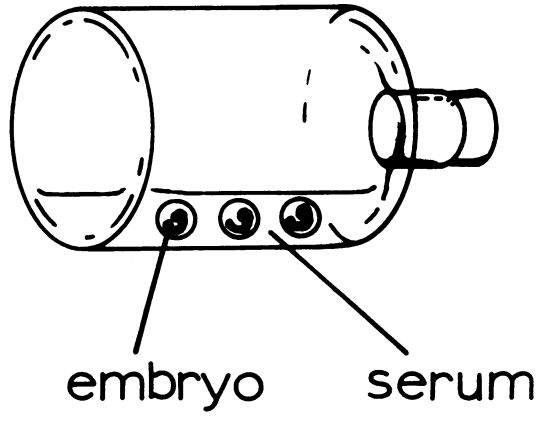

FIGURE 4. Culture bottle 3 cm diameter ×4 cm.

difference was found between the embryos grown *in vitro* and *in vivo* as regards either protein content (Fig. 5) or stage of development (Fig. 6).

The results at 48 hr were more variable. If incubation of the 32-hr cultures was continued to 48 hr without any other treatment, the embryos had

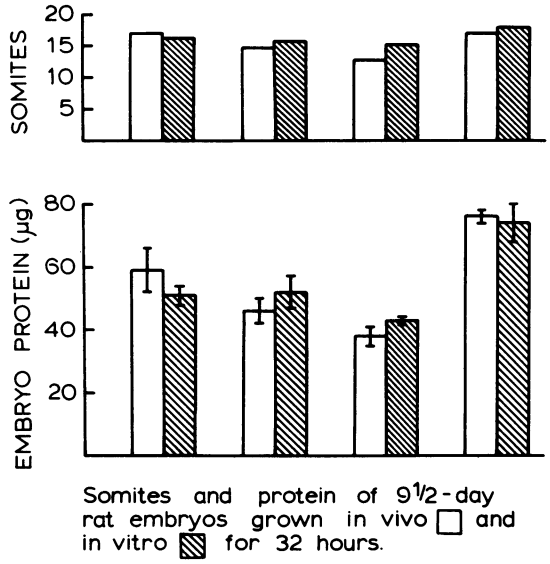

FIGURE 5. Comparison of growth *in vivo* and *in vitro* after 32 hr of the embryos from four rats. Each pair of rectangles shows the means and standard errors of the two groups of four embryos from each rat (see Fig. 2).

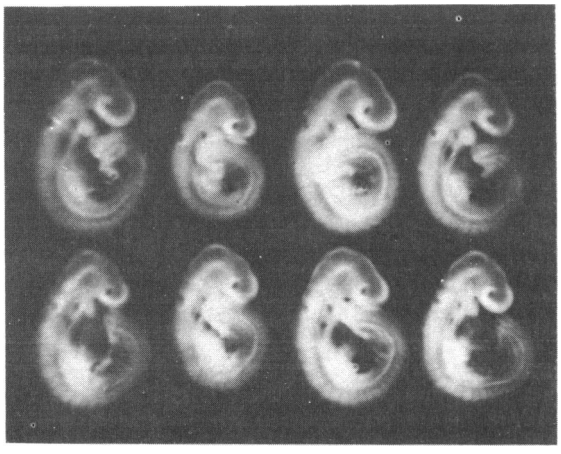

FIGURE 6. Embryos grown for 32 hr *in vivo* (upper row) and *in vitro* (lower row). All eight embryos are from the same rat. Photographed after removal of the embryonic membranes.

only about 65% of the protein content of littermates *in vivo* (Fig. 7, series A). However, if the culture bottles were regassed at 32 hr with 20% O_2-5% CO_2-75% N_2, final embryo protein *in vitro* rose to over 80% of that *in vivo* (series C). Further gains could be made by raising the O_2 level at 32 hr to 40% and by transferring the embryos during the culture period to a larger bottle with fresh serum. The protein content of the embryos *in vitro* then became very similar to that of the controls *in vivo* (series D, E), and a corresponding similarity was found in overall appearance (Fig. 8) and in the histology (*6*).

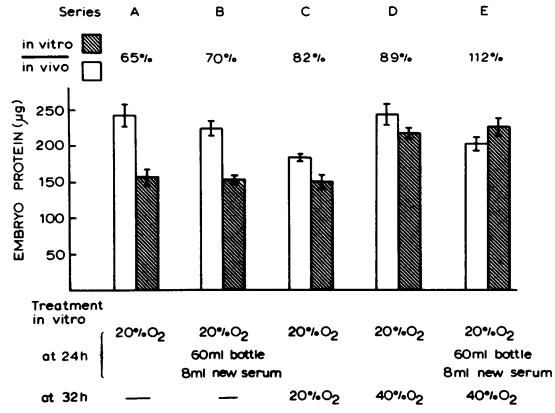

FIGURE 7. Comparison of final embryo protein *in vivo* and *in vitro* after 48 hr and under five different culture treatments. Each rectangle in the histogram shows the mean and standard error of 12 embryos.

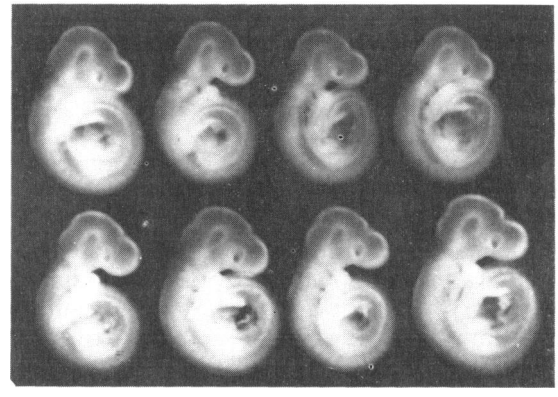

FIGURE 8. Embryos grown for 48 hr *in vivo* (upper row) and *in vitro* (lower row). All eight embryos are from the same rat. Photographed after removal of the embryonic membranes. ×6.

I have described this experiment in some detail because I hope it will remove two of the main objections that are often raised against culture methods for embryos: that the techniques are complicated and the cultured embryos abnormal. I believe that for the rat embryo we now have a very simple method, suitable for routine use with large numbers of embryos, which will grow embryos that resemble very closely indeed those *in vivo*. The period over which normal growth rates can be maintained extends from the primitive

streak to early limb-bud (about 25-somite) stages. The culture method will also support further development to give larger and more advanced embryos (Fig. 9), but the growth rate is then somewhat slower than normal.

To illustrate some of the possible applications of the method, I will briefly outline three studies that we are currently engaged in at Cambridge.

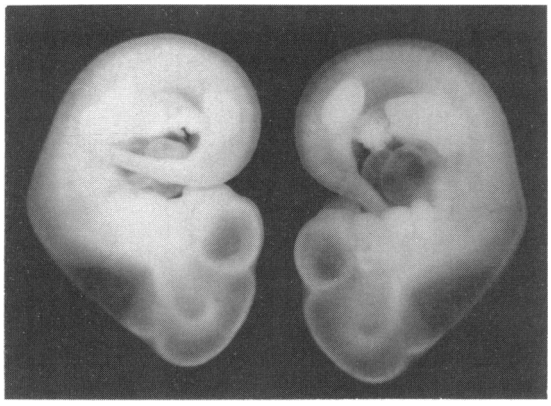

FIGURE 9. Two rat embryos explanted at 9½ days gestation (head-fold stage) and grown in culture for 95 hr. × 7.

Hyperthermia

Temporary elevation of the body temperature, resulting from climatic influences or infection, occurs in many mammals. Such hyperthermia is often harmless, but in the pregnant female it may be teratogenic, with particularly damaging effects on the brain of the developing embryo. The studies that have been made on experimentally induced hyperthermia and teratogenesis in animals have recently been reviewed (*9*) and suggest that hyperthermia may also be teratogenic in man. In most of these experiments on pregnant animals the period of exposure to high temperature was rather short, often only 40-60 min, and the temperature experienced by the embryos usually varied during treatment and could not be controlled precisely.

In culture, the embryos can be exposed to raised temperatures for longer and under closely specified conditions. It also becomes possible to distinguish direct effects of hyperthermia from those that may result indirectly from changes in the maternal metabolism. We have examined the effects of hyperthermia on rat embryos explanted at head-fold stage and grown in culture for 46-47 hr (10). A comparison was made of the development of embryos exposed to control temperature (37-38°C), to 40°C, and to 41°C throughout the culture period. At the control temperature, the embryos developed normally and formed about 23-26 somites with brain and spinal cord, optic and otic vesicles, anterior limb buds, and a well developed heart and blood circulation. At 40°C, overall growth was similar to that of the controls, although a smaller proportion of these embryos had a good blood circulation at the end of the culture, and about half showed small developmental abnormalities, including a few with slight microcephaly. In contrast, the embryos incubated at 41°C were very severely affected. Final protein content was much less than that of embryos incubated at either 37-38°C or 40°C. More than half the embryos in this group were microcephalic and almost as many had enlarged hearts and edema of the pericardium. Commonly, the posterior part of the trunk was sharply bent back on the anterior, with fusion of the anterior and posterior neural folds.

When the embryos were exposed to 41°C for shorter periods (12 or 24 hr), many abnormalities of development still occurred, but they were less severe. The results suggested that the first few hours of day 11 (neural folds and earliest somites) may be a particularly sensitive period. A notable feature of the results is the very small temperature rise—only 2-3°C above the normal—needed to produce abnormalities of development. Such small rises are well within the range commonly experienced during fever and suggest that studies of this kind on the embryos of laboratory animals in culture may be relevant to problems of hyperthermia and teratogenesis in man.

Heat-Inactivated Serum

The nutrient medium routinely used in our culture system is homologous serum. For older embryos (e.g., rat embryos over 13 days), development may be as good or better in serum diluted with synthetic media. But at the earlier stages of organogenesis, maximum growth of the embryos is obtained in undiluted serum.

Both the embryos and the culture serum that we are currently using are obtained from the CFHB strain of rats. It appears to make no difference whether the serum is obtained from pregnant female, nonpregnant female or male rats. However, it is important that the serum be heat-inactivated (i.e., heated to 56°C for 30 min) before use. In one study (*5*) we compared the

growth of 48 rat embryos explanted at head-fold stage and grown in heat-inactivated serum with the same number grown in untreated serum. Those in the untreated serum showed many more abnormalities of development (Fig. 10), were often retarded, and the average protein content was reduced by 24%.

The heat-inactivation treatment is known to inactivate most of the complement in rat serum (*8*). This suggests that the relatively poor development of the embryos in the untreated serum may result from a complement-dependent immune reaction. We are now investigating this by comparing growth of littermate embryos in serum from different individuals and different strains of rat, and, in a further series of experiments, by adding known amounts of complement to the heat-inactivated serum. But the possibility remains at present that the heat treatment may be affecting any of the enzymes or other proteins in the serum, and the effects that we have observed on cultured embryos could result from changes in the serum other than, or in addition to, those of the complement.

Oxygen

Previous work has shown that very high oxygen concentrations (e.g., gas mixtures containing 95% or more of oxygen) are rapidly lethal to the younger embryos (*2,11*). Recently we have found that a rather specific abnormality of development is caused by 20% O_2 in the gas phase of the culture (*5*). Head-fold embryos grown in cultures with 20% O_2-5% CO_2-77% N_2 were compared with those in 5% O_2-5% CO_2-90% N_2. No significant difference was found in the development of the blood circulation, yolk sac and allantois, in the rotation of the embryo to the fetal position, in the number of new somites formed or in the amount of protein synthesized. But a striking difference was noted in the development of the neural tube, particularly in the embryos grown in untreated (not heat-inactivated) serum. Of these 15/24 showed a failure of closure of the neural tube in 20% O_2 but only 4/24 in 5% O_2. Such failures of tube closure were fewer in the embryos grown in heat-inactivated serum, but whereas none occurred in 24 embryos grown in 5% O_2, 2/24 were found in 20% O_2. Usually only the brain was affected, and the malformation varied from a small hole in the roof of the hind brain to wide open neural folds extending for most of the length of the head (Fig. 11), but in a few embryos part or all of the spinal cord also remained open.

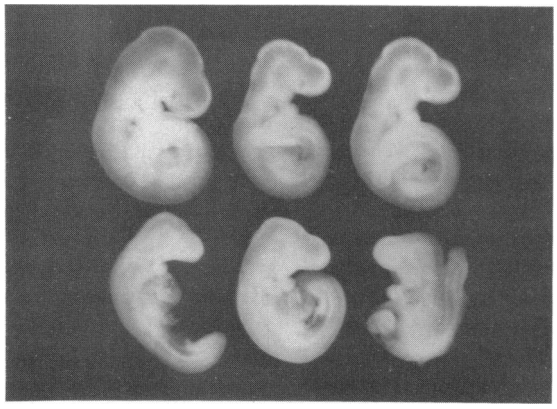

FIGURE 10. Six embryos from the same rat, explanted at 9½ days gestation and grown for 48 hr in "heat-inactivated" serum (top row) and untreated serum (bottom row). The embryos at bottom left and right have failed to adopt the normal fetal position; in the one on the right the anterior and posterior parts of the trunk have fused together dorsally. ×6.

Calculation shows that the oxygen tension in the culture bottles with 5% O_2 was about 40 mm Hg. This is close to the oxygen tension found in the uterine vein or abdominal vena cava (*12,13*) and is probably adequate for normal growth of the embryo only because the embryo at this stage can obtain much of its energy needs from anaerobic glycolysis (*11*). The culture bottles with 20% O_2 have an oxygen tension of about 160 mm Hg. This is higher than the 80-100 mm Hg usual for arterial oxygen, but not higher than the oxygen tension of air. It is interesting that oxygen at this level is apparently teratogenic, and the result may be compared with that of Ferm (*14*) who reported exencephaly, spina bifida, and other fetal abnormalities in pregnant golden hamsters exposed to hyperbaric oxygen at stages of gestation equivalent to those of our rat embryos.

General Comments

The three examples given above illustrate how the present culture methods can be applied to the study of teratogenic effects resulting from variations in the environmental temperature, the nutrient medium and the respiratory gases. Other studies for which embryo culture methods have been used include the action of specific antibodies (*15,16*), of vitamins (*17,19*), of clotting factors (*8*), and of cardioactive drugs (*20*), the pathways of energy metabolism in the embryo (*11*), the mechanisms of axial rotation (*21,22*), and the

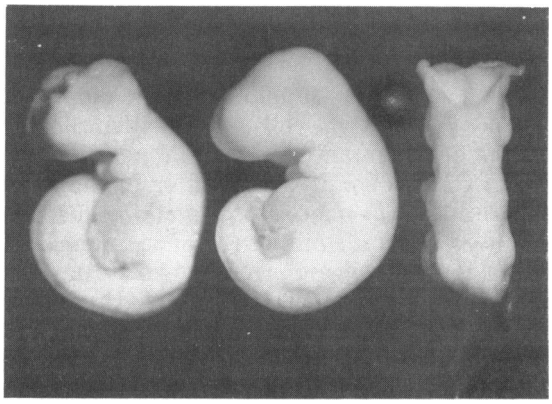

FIGURE 11. Failure of closure of the head folds in embryos grown in 20% O_2 throughout the 48-hr culture period. Photographed after fixation. × 8.

functions of the embryonic membranes (*23*). Limitations of the present culture methods are that they support entensive organogenesis only in the embryos of rodents, and development cannot be continued to the late stages of gestation (the limit is about day 15 in the rat, equivalent to about 6-7 weeks of pregnancy in man). But the methods are simple and reliable and allow very close observation of development and precise control of test conditions. They can distinguish clearly between direct effects of an agent on the embryo and effects mediated by the maternal metabolism. They support development during the whole period of major organogenesis, and for the earlier part of this period the rates of growth and differentiation in culture are now indistinguishable from those *in vivo*.

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